

# Caeruloplasmin isoforms in Wilson's disease in neonates

G F E Chowrimootoo, H Scowcroft, Carol A Seymour

## Abstract

**Aim**—To investigate the neonatal diagnosis of Wilson's disease from caeruloplasmin isoforms in cord blood.

**Methods**—Serum caeruloplasmin isoforms were measured in 5–10 ml cord blood from 10 fresh umbilical cords using sodium dodecyl polyacrylamide gel electrophoresis (SDS PAGE) and western blotting and analysed by densitometry. Total caeruloplasmin concentrations were determined by nephelometry and caeruloplasmin oxidase by p-nitrophenyldiamine.

**Results**—Although total caeruloplasmin concentrations are reduced in neonates, the plasma isoform was significantly reduced or absent in patients with Wilson's disease. Sera from healthy neonates and from those with Wilson's disease had reduced biliary isoforms.

**Conclusion**—Identification of caeruloplasmin isoforms may be a marker for Wilson's disease in neonates.

(Arch Dis Child Fetal Neonatal Ed 1998;79:F198-F201)

**Keywords:** caeruloplasmin isoforms; Wilson's disease; copper excretion

Although copper is an essential trace metal in the human body, and a component of many enzyme systems, dietary copper intake exceeds requirements. Copper homeostasis is controlled by biliary excretion of copper into the bile rather than by intestinal absorption.<sup>1</sup> Wilson's disease is an inherited disorder of copper metabolism, with impairment of biliary copper excretion, resulting in copper accumulation in the liver and consequent damage. There is also a variable reduction in, or absence of, circulating caeruloplasmin, the major copper transporting protein in plasma, which has until recently been difficult to explain in terms of the pathophysiology of this disease.<sup>2</sup>

Analysis of the Wilson's disease gene now mapped to chromosome 13q 14.3, has shown a 54% homology with the ATP7A gene that is associated with the genetic copper deficiency disorder, Menke's disease. Thus there may be common copper transporting proteins. The association between the membrane bound copper proteins and the circulating transporter, caeruloplasmin, is now of interest.

Fetal copper metabolism is different from that of the adult,<sup>3,4</sup> with the fetal liver tolerating up to 20 times the adult liver copper concentration without damage. This apparent similarity to Wilson's disease has led to the suggestion that in this disease there is a failure to change from the neonatal mode of copper metabolism

to the adult mechanism.<sup>5,6</sup> In both Wilson's disease and the normal fetus biliary copper excretion is greatly reduced, with low plasma copper and absent or low plasma caeruloplasmin concentrations. Hepatic copper accumulation in both situations seems to result from the biliary rather than altered plasma caeruloplasmin concentration as hereditary acaeruloplasminaemia is not associated with hepatic copper accumulation or liver damage.<sup>7</sup>

Caeruloplasmin, a single chain glycoprotein (132 kiloDaltons) synthesised in the liver, is found in the  $\alpha_2$ -globulin fraction of mammalian plasma. Caeruloplasmin oxidase is a measure of its functional activity as an antioxidant. Irrespective of a reduction in, or absence of, circulating caeruloplasmin, this protein is synthesised in the liver in the normal way in Wilson's disease, and there are two molecular isoforms,<sup>8</sup> one predominating in bile (125 kiloDaltons) and the other in plasma (132 kiloDaltons). The biliary form is always absent in Wilson's disease bile and may be important in copper excretion. Iyengar *et al*<sup>9</sup> first noted an absence of cross reacting material to caeruloplasmin antibodies in Wilson's disease bile, while the bile of normal subjects contained enough copper in caeruloplasmin molecules to account for copper balance regulation. They proposed that the Wilson's gene may concomitantly affect the appearance of caeruloplasmin in the blood and into the bile, thus simultaneously accounting for both defects<sup>9</sup>. However, the precise mechanism by which the caeruloplasmin isoforms are transported to plasma or to bile is still uncertain. Our studies suggest that caeruloplasmin with its copper transporting function may be involved in biliary copper excretion, by binding to the putative copper transporter protein, which is defective in Wilson's disease.<sup>8,9</sup>

## Methods

Cord blood (5–10 ml) was obtained from 10 fresh umbilical cords (full term gestation) using sterile 27 mm gauge needles. Blood was placed immediately in a sterile tube (without additives) and allowed to clot at 4°C, before centrifuging for 15 minutes at 6000 rpm on a bench centrifuge. Other venous blood samples were collected from healthy subjects (age range 25–42 years) or known Wilson's disease patients (age range 28–47 years) using standard procedures during routine clinical investigations. All samples were stored at -20°C for up to three months. These studies had the approval of the local ethics committee.

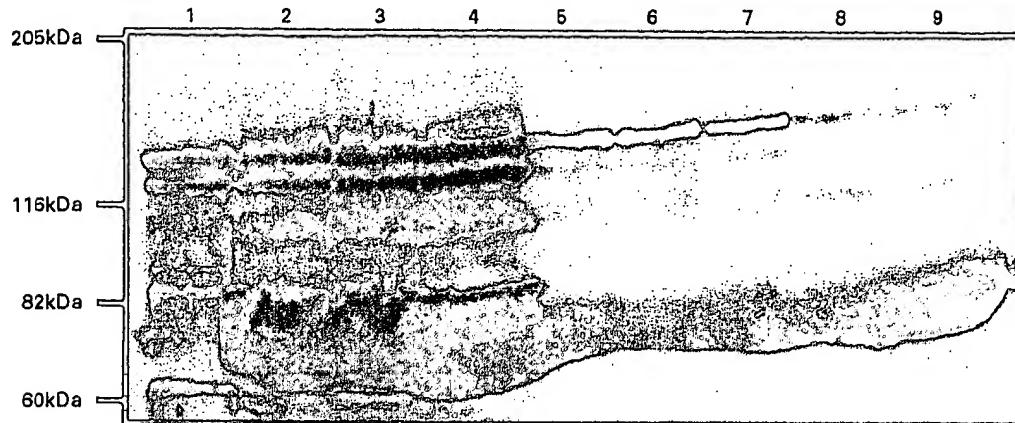
Bis/Acrylamide for SDS-PAGE was purchased premixed as Protogel from National Diagnostics. Affinity purified polyclonal sheep

Division of  
Cardiological Sciences  
(Metabolic Medicine),  
St George's Hospital  
Medical School,  
Cranmer Terrace,  
London SW17 0RE  
G F E Chowrimootoo  
H Scowcroft  
C A Seymour

Correspondence to:  
Professor Carol A Seymour.  
Email: cseymour@  
sghms.ac.uk

Accepted 30 March 1998

## BEST AVAILABLE COPY



**Figure 1** Molecular forms of caeruloplasmin in serum detected by western blotting. Representative blot shows serum samples (200 µg protein) subjected to electrophoresis on SDS-PAGE, transferred to a nylon membrane by western blotting and detected with anti-human caeruloplasmin antibody. Lane 1: purified serum caeruloplasmin; lanes 2-4: normal adult sera; lanes 5-7: cord sera; lanes 8-9: Wilson's disease sera. Neonatal sera have similarities to normal adult sera in respect of plasma caeruloplasmin (132 kiloDaltons) and to Wilson's disease sera in respect of biliary caeruloplasmin (125 kiloDaltons). In Wilson's disease both caeruloplasmin forms are clearly and significantly reduced.

anti-human caeruloplasmin antibody (peroxidase conjugate) was obtained from Serotec, Oxford. Hybond-C Super and enhanced chemiluminescence (ECL) western blotting detection reagents were provided by Amersham International, Hertfordshire, UK. All other chemicals were from either Sigma, Poole, Dorset, or BDH, Dagenham, Essex, UK.

Serum samples were subjected to 7.5% SDS-PAGE, as described before.<sup>10</sup> The gel was blotted on to a Hybond-C super membrane using a semi-dry blotter (BioRad Transblot-SD) for 90 minutes at constant current of 1 mA/cm<sup>2</sup>.

The blot was blocked in 5% milk solution (containing 50 mM Tris/150 mM NaCl and 0.02% NaN<sub>3</sub>, pH 7.4) for 2 hours and incubated for 3 hours with horseradish peroxidase-conjugated sheep anti-human caeruloplasmin. The blot was washed several times in Tris/NaCl buffer and caeruloplasmin was detected using the ECL method described by Amersham. Serum caeruloplasmin concen-

trations were measured on all samples using a standard laboratory nephelometric method.<sup>11</sup>

For densitometric analysis, blots were scanned on a Microtek ScanMaker IISP scanner and analysed using NIH image Blots 1.52 (from National Institute of Health Research Services Branch, Bethesda, MD), as described before.<sup>12</sup>

Caeruloplasmin oxidase activity was measured, as described before.<sup>13</sup> Serum samples containing 2 mg protein were added in duplicate to plastic cuvettes containing 0.6 ml of 0.1M acetate buffer (pH 6.0), 0.3 ml 0.25% p-Phenylenediamine (PPD) in 0.1M acetate buffer, which had been equilibrated at 37°C for 5 minutes. Blank samples containing 2 mg plasma protein, 0.3 ml 0.1M acetate buffer, 0.3 ml 0.1% NaN<sub>3</sub> in acetate buffer, and 0.3 ml 0.25% PPD in acetate buffer were prepared for each test sample. The reaction mixtures were incubated at 37°C, and the absorbance read at 530 nm after 10 and 40 minutes.

Caeruloplasmin oxidase activity was calculated in terms of oxidase units, where one oxidase unit =  $(\text{Abs}_{40\text{min}} - \text{Abs}_{10\text{min}}) \times 1000$ .

## Results

As expected both molecular forms of caeruloplasmin (132 kiloDaltons and 125 kiloDaltons) were present in sera from normal adult subjects (fig 1, lanes 2-4). By comparison, cord samples showed substantial amounts of 132 kiloDalton caeruloplasmin (fig 1, lanes 5-7) similar to adults but with a greatly reduced 125 kiloDalton caeruloplasmin expression and a profile similar to that of sera of patients with Wilson's disease (fig 1, lanes 8-9). Densitometric analysis of these western blots is summarised in table 1.

Caeruloplasmin oxidase activity in cord samples was similar to that in normal control adult subjects (table 2), but, as expected in Wilson's disease, caeruloplasmin oxidase activity is negligible or absent.

Table 3 shows total caeruloplasmin measured by standard method comparing cord

**Table 1** Densitometric analysis of molecular forms of caeruloplasmin in serum samples from normal adult cord and Wilson's disease samples

	Normal adult	Cord samples	Wilson's disease
132 kDa	171.6 (6.5) (5)	127.8 (8.3) (5)	19.8 (17.7) (5)
	161.2-179.5	117-139.1	0.3-41.9
125 kDa	159.8 (14.4) (5)	47.6 (26.8) (5)	2.1 (17.7) (5)
	137.5-170.7	19.5-90.1	0-8.1

Molecular forms of caeruloplasmin are shown in OD units in sera from normal controls, cord blood and Wilson's disease samples. Densitometry was as previously described.<sup>11</sup> Data are shown as mean (SEM), with number of samples and range below.

**Table 2** Caeruloplasmin oxidase activity in normal adult cord and Wilson's disease serum samples

Sample (number)	Average oxidase activity (units*)
Control (4)	210.8 ± 49.3
Cord blood (9)	187.4 ± 26.0
Wilson's disease (4)	7.8 ± 2.8

\* Caeruloplasmin oxidase activity is expressed as units/2 mg protein.

Table 3 Caeruloplasmin measurement in cord and normal adult serum

Sample (number)	Mean caeruloplasmin (g/l) (SEM)	Range
Cord (9)	0.152 (0.02)	0.09-0.27
Normal adult (7)	0.264 (0.02)	0.20-0.35

sera in adult control subjects and those with Wilson's disease. Total caeruloplasmin was reduced in cord samples in accord with previous studies.<sup>1</sup>

### Discussion

In contrast to adults, neonates tolerate a higher hepatic copper concentration which occurs during gestation.<sup>14</sup> Both plasma copper and caeruloplasmin concentrations are low at this developmental stage. Caeruloplasmin is synthesised in the liver and secreted into the serum as the major copper transporting protein,<sup>3</sup> and although its main role is in copper transport to extrahepatic tissues, it has other important functions in the body.<sup>3</sup> Its precise role in copper metabolism and homeostasis is unclear.<sup>15</sup> Neonatal hepatic caeruloplasmin synthesis occurs after birth and is associated with a gradual rise in plasma caeruloplasmin and copper concentrations. Normalisation of hepatic copper concentrations occurs once copper excretion is initiated. Thus three to six months after birth, a concomitant reduction in hepatic copper content occurs towards normal adult concentrations. We have already suggested that biliary copper excretion in adults may involve caeruloplasmin predominantly in the 125 kiloDalton molecular isoform in a process that is defective in Wilson's disease, and which may account for hepatic copper accumulation.<sup>7,8</sup>

In this study, the concentration of 132 kiloDalton plasma caeruloplasmin found in cord blood was not significantly different from that in normal control adult serum; it was, however, markedly reduced in Wilson's disease samples, clearly distinguishing between normal neonates and those with Wilson's disease. The reduced expression of the 125 kiloDalton form in cord samples was similar to that found in untreated adults with Wilson's disease (fig 1) (table 1).

These observations support our suggestion that this lower molecular weight form of caeruloplasmin is required for biliary excretion. In neonates, the absence of this form would explain the increased hepatic copper concentration consequent on immature biliary excretion. This mimics the situation in Wilson's disease.

In this study caeruloplasmin oxidase activity (the functional aspect of caeruloplasmin) in cord samples was comparable with normal adult oxidase activity and significantly higher than in Wilson's disease (table 2). The presence of oxidase activity indicates that there is copper incorporation (holocaeruloplasmin). We have also shown that copper is normally incorporated into caeruloplasmin in Wilson's disease.<sup>9</sup> However, this reduction in oxidase activity occurs not only in Wilson's disease, but also in Menke's disease (genetic copper

deficiency) and in other serious liver disorders affecting protein synthesis, and is therefore not specific for diagnosis of Wilson's disease, but can be used to confirm western blotting analysis.

This argues against a failure of copper incorporation accounting for hepatic copper accumulation in neonates and more for a failure of biliary copper transport. The lack of a biliary caeruloplasmin component could, however, explain hepatic copper retention, as in Wilson's disease. This finding further supports our previous proposal that the defect in Wilson's disease is most likely to involve biliary caeruloplasmin (transporting copper) bound in some way to the ATB7B copper transporter.<sup>15</sup> A reduction in caeruloplasmin that was previously noted in neonatal serum,<sup>4</sup> and in this study (table 3), is attributed predominantly to a lack of the 125 kiloDalton isoform. In the past it has been difficult to detect Wilson's disease in neonates, as total plasma copper and caeruloplasmin are both reduced in neonates. The clear difference in expression of the 132 kiloDalton caeruloplasmin isoform in cord blood and Wilson's disease samples, as shown by western blotting (fig 1 and table 2), could be used to distinguish Wilson's disease in the neonatal period. Early diagnosis of Wilson's disease would be likely to prevent complications such as haemolysis, cirrhosis, and permanent brain damage,<sup>16,17</sup> because treatment with a chelating agent to remove copper could be commenced before any tissue damage.

In summary, using western blotting of cord blood, we have shown that the reduced plasma caeruloplasmin in neonates is due to reduced expression of biliary (125 kiloDaltons) caeruloplasmin which is also defective in Wilson's disease. In both situations hepatic copper accumulation occurs which is later compensated in neonates without Wilson's disease in the six months after birth by synthesis of caeruloplasmin and unloading of hepatic copper.

The presence of oxidase activity in umbilical cord sera and impairment of biliary copper excretion in neonates further supports the original proposal of Iyengar *et al* and our concepts that caeruloplasmin has a major role in biliary copper excretion. It also supports our finding that caeruloplasmin in Wilson's disease incorporates copper normally.<sup>9</sup> The clear difference in expression of caeruloplasmin between cord and Wilson's disease blood samples by western blotting may be useful in the diagnosis of Wilson's disease in the first 3 to 6 months after birth, where we would expect absent or undetectable concentrations of both isoforms. This would be feasible as western blotting requires only a few microlitres of serum and could be made even more user friendly by extraction from filter paper, thus avoiding previous sampling and diagnostic problems. This finding is being developed further using Guthrie card blood spots to pick up pre-symptomatic Wilson's disease.<sup>18</sup> This would enable the diagnosis of Wilson's disease to be made before substantial tissue damage.

We thank Professor George Brewer, Department of Human Genetics, University of Michigan, for helpful comments, Miss Caroline Cooper for typing the manuscript, and South Thames Health Authority Research and Development Grants (CAS), for their support for this work.

- 1 Sass-Kortsak A, Bearn AG. Copper metabolism. *Adv Clin Chem* 1965;8:1-67.
- 2 Danks DM. Disorders of copper transport. In: Scriver CR, Beaufet AL, Sly WS, Calle D, eds. *Metabolic and molecular basis of inherited disease*. vol 1; 7<sup>th</sup> edn. New York: McGraw-Hill, 1995; Part 10 (metals): 2211-37.
- 3 Gutteridge JMC, Stocks J. Caeruloplasmin: physiological and pathological perspectives. *Crit Rev Clin Lab Sci* 1981;14:257-329.
- 4 Aggett PJ. Aspects of neonatal metabolism of trace metals. *Acta Paediatr Suppl* 1994;402:75-82.
- 5 Epstein O, Sherlock S. Is Wilson's disease caused by a controller gene mutation resulting in the perpetuation of the foetal mode of copper metabolism into childhood? *Lancet* 1981;i:303-5.
- 6 Srai SK, Burroughs AK, Wood B, Epstein O. The ontogeny of liver copper metabolism in the guinea pig: clues to the etiology of Wilson's disease. *Hepatology* 1986;6:427-32.
- 7 Davis W, Chowrimootoo GFE, Seymour CA. Defective biliary excretion in Wilson's disease: the role of caeruloplasmin. *Eur J Clin Invest* 1996;26:893-901.
- 8 Iyengar V, Brewer GJ, Dick RD, Owyang C. Studies of cholecystokinin-stimulated biliary secretions reveal a high molecular weight copper binding substance in normal subjects that is absent in patients with Wilson's disease. *J Lab Clin Med* 1988;111:267-74.
- 9 Chowrimootoo GFE, Hamed HA, Seymour CA. New insights into the pathogenesis of copper toxicosis in Wilson's disease. *Biochem J* 1994;315:851-5.
- 10 Laemmli UK. Cleavage of structural proteins during the assembly of the head of a bacteriophage. *Nature* 1970; 227:680-5.
- 11 Chan A, Wong F, Arumanayagam M. Serum ultrafiltrable copper, total copper and caeruloplasmin concentrations in gynaecological carcinomas. *Ann Clin Biochem* 1993;30:545-9.
- 12 Friedland JS, Remick DG, Shattock R, Griffin GE. Secretion of interleukin- $\beta$  following phagocytosis of mycobacterium tuberculosis by human monocyte cell lines. *Eur J Immunol* 1992;22:1373-8.
- 13 Henry RJ, Chiamoris N, Jacobs SL, Seaglove M. Determination of caeruloplasmin oxidase activity in serum. *Proc Soc Exp Med Biol* 1960;104:620-4.
- 14 Dorea JG, Brito M, Araujo MOG. Concentration of copper and zinc in liver of fetuses and infants. *J Am Coll Nutr* 1987;6:491-5.
- 15 Frieden E. Perspectives in copper biochemistry. *Clin Physiol Biochem* 1986;4:11-19.
- 16 Sternlieb I, Scheinberg IH. Prevention of Wilson's disease in asymptomatic patients. *N Engl J Med* 1968;278:352-9.
- 17 Walsh JM, Yealland M. Not Wilson's disease: a review of misdiagnosed cases. *Q J Med* 1995;88:55-9.
- 18 Guthrie RE, Susi A. A simple phenylalanine method of detecting phenylketonuria in large populations of infants. *Paediatr* 1963;32:338-43.